Role Of Conserved Cysteines In The Alphavirus E3 Protein

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ABSTRACT

Alphavirus particles are covered by 80 glycoprotein spikes essential for viral entry. Spikes consist of the E2 receptor binding protein and the E1 fusion protein. Spike assembly occurs in the ER where E1 associates with pE2, a precursor containing E3+E2 proteins. E3 is a small, cysteine rich, extracellular glycoprotein that mediates proper folding of pE2 and its subsequent association with E1. In addition, cleavage of E3 from the assembled spike is required to make the virus particles efficiently fusion competent. We have found that the E3 protein in Sindbis contains one disulfide bond between residues Cys19 and Cys25. Replacing either of these two critical cysteines resulted in mutants with attenuated titers. Replacing both cysteines with either alanine or serine resulted in double mutants that were lethal. Insertion of additional cysteines based on E3 proteins from other alphaviruses resulted in either sequential or nested disulfide bond patterns. E3 sequences that formed sequential disulfides yielded virus with near wild-type titers while those that contained nested disulfide bonds had attenuated activity. Our data indicate that the role of the cysteine residues in E3 is not primarily structural. We hypothesize that E3 has an enzymatic or functional role in virus assembly and these are further discussed.

INTRODUCTION

Alphaviruses are members of the Togaviradae family and are single-stranded, positive sense RNA, enveloped viruses (17). The lipid membrane of the virus has 80 glycoprotein spikes which are required for viral entry. Each spike is comprised of three copies of a heterodimer which consists of the E2 and E1 proteins (22, 54). E2 and E1 are each glycoproteins with a single transmembrane helix that traverses the host-derived lipid bilayer. E2 interacts with the nucleocapsid core at the C-terminus (12, 16, 27, 43) and contains the receptor binding site at the N-terminus (5, 21, 45). E1 is the viral fusion protein responsible for mediating fusion between the virus membrane and the host cell membrane during an infection (13, 39, 47). Specific interactions in both the ectodomain and transmembrane regions are critical for heterodimer formation (30, 35, 46, 54). The assembly of each heterodimer, its subsequent assembly into a spike and the interaction of the cytoplasmic tail of the spike with the nucleocapsid core are all essential for the efficient production of infectious particles.

Glycoprotein spike assembly requires four structural proteins, E3, E2, 6K, and E1, which are expressed as a single polyprotein. E3 is a small 64 amino acid protein (Sindbis virus numbering) and contains a signal sequence that translocates the protein into the ER (3, 4, 15). Early in translation, glycosylation of N14 (Sindbis numbering) occurs and this promotes E3's release from the ER membrane into the lumen. As a result, the signal sequence is not cleaved from the E3 protein (14). Cellular enzymes cleave the polyprotein to yield pE2 (an

uncleaved protein consisting of E3+E2), 6K, and E1 (23, 55) proteins. In the ER, E1 is found in several conformations, only one of which will form a functional heterodimer with pE2 allowing its transport to the Golgi (1, 2, 6, 7, 36). After pE2-E1 heterodimerization, self-association between three heterodimers occurs and each individual spike is formed (25, 26, 36). As observed with Semliki Forest virus, disulfide bonds reshuffle within pE2 during protein folding (34), possibly forming intermolecular disulfide bonds between E3 and E2 residues. However, no intermolecular disulfide bonds between pE2 and E1 have been identified (34). Once the viral spikes have been assembled, they are transported to the plasma membrane (11) and are thus exposed to sub-cellular changes of pH, from pH 7.2 in the ER to pH 5.7 in the vesicles constitutively transporting the spikes to the plasma membrane. In the trans-Golgi, the E3 protein is cleaved from pE2 by the cellular protein furin (18, 44, 55). E3 remains non-covalently attached to the released virus particle, while in other species E3 is found in the media of virus infected cells (32, 49).

E3 is required for efficient particle assembly, both in mediating spike folding and spike activation for viral entry. When an ER signal sequence was substituted for the E3 protein, heterodimerization of pE2 and E1 was abolished (26). Furthermore, when E2 and E1 were expressed individually, low levels of E2 were transported to the cell surface while E1 remained in the ER suggesting that heterodimerization with pE2 is necessary for E1 to be transported to the cell surface (24, 26, 46). These results are consistent with E3 playing a critical role in mediating the folding of pE2 and the association of pE2 and E1 proteins during

spike assembly (7, 38). In viruses where the furin cleavage site was mutated, the virus particles were correctly assembled but severely reduced in infectivity, presumably because the fusion protein was unable to dissociate from pE2 and initiate fusion (44, 55).

A comparison of an amino acid sequence alignment of E3 proteins from different alphaviruses (Figure 1) shows the E3 protein is a small protein with 4 conserved cysteine (Cys) residues. A subset of E3 proteins contains an additional 2 Cys residues in a narrow cysteine/proline rich region, PPCXPCC (Figure 1). We have purified recombinant E3 protein from Sindbis virus and have determined that a disulfide bond is present and, furthermore, these Cys residues are important in virus assembly. Within the alphavirus E3 proteins, we have identified a region that is important for mediating spike transport to the plasma membrane and thus is critical for spike assembly.

MATERIALS AND METHODS

Viruses and cells. All virus mutations were made in a full length cDNA TE12 clone of Sindbis (28). BHK-21 cells (American Type Tissue Culture, Rockville, MD) were grown in minimal essential media (MEM, Gibco Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) at 37°C in the presence of 5% CO_2.

E3 cloning, expression and purification. E3 from Sindbis virus was cloned into a SUMO expression vector (courtesy of Dr. Thomas Bernhardt, Harvard

Medical School, Boston, MA) and transformed into Rosetta gami 2 (Novagen, Darmstadt Germany) chemically competent cells. For a large scale expression of the SUMO-E3 fusion protein, referred to as SUMO-E3, cells were grown in Terrific Broth medium supplemented with ampicillin at a final concentration of 100 µg/ml and chloramphenicol at a final concentration of 37 µg/ml at 37°C. When OD_{600} reached 0.4-0.6, cells were induced with IPTG at a final concentration of 1mM, shifted to 16°C, and grown for an additional 18 hours. Cells were pelleted at 4500 × *g* for 15 min at 4°C. Cell pellets were resuspended in 5 ml Buffer A (20 mM Phosphate pH 8, 300 mM NaCl, 10 mM imidazole) per 1 g cell pellet. A protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN) was added. Cells were lysed with two passages through a French pressure cell at 12,000 lb/in^2 (SLM-Aminco, Urbana, Ill.) or three passages through a continuous flow microfluidizer (MicroFluidics, Taylorsville, UT). Unlysed cells and insoluble material were pelleted at 125,000 × *g* for 30 min. The clarified lysate was filtered using a 0.2 µm syringe filter and loaded onto a HisTrap FF Crude column (1 ml) (GE Healthcare, Piscataway, NJ). Protein was eluted by a step gradient made from Buffer A and Buffer B (20 mM Phosphate pH 8, 300 mM NaCl, 300 mM imidazole). Fractions corresponding to SUMO-E3 were concentrated and buffer exchanged into 20 mM Phosphate pH 8 and 300 mM NaCl using 3K Amicon Ultra concentrators (Millipore, Billerica, MA) at 5000 × *g* at 4°C. The SUMO-E3 protein was digested with SUMO protease (courtesy of Dr. Thomas Bernhardt, Harvard Medical School, Boston, MA) for 48 h at 4°C and loaded onto HisTrap HP (1 ml). Fractions containing E3 elute in the flow through while SUMO and the

SUMO protease remain bound to the column. Isolated E3 proteins were concentrated as described above. Isolated E3 protein concentration and purity was determined by standard Bradford assay and 10-20% Tris-Tricine SDS-PAGE.

Circular dichroism. Circular dichroism (CD) measurements were carried out in a J-715 Circular Dichroism spectropolarimeter (JASCO), which is equipped with a PTC-343 Peltier-type cell holder for temperature control. E3 samples were prepared in 20 mM phosphate buffer pH 8 and 100 mM NaF. CD spectra from 190 nm to 260 nm were recorded in a 1 mm path length cuvette at 10°C.

Gel Filtration. Purified E3 protein was loaded onto a Superdex peptide HR 10/30 column (GE Amersham, Piscataway, NJ) equilibrated in 20 mM Phosphate pH 8 and 300 mM NaCl. The sample was eluted from the column at 0.5 ml/min and fractions were analyzed using 10-20% Tris-Tricine SDS-PAGE.

Expression of ^{15}N E3 and NMR Spectroscopy. To produce uniformly ^{15}N-labeled E3, SUMO-E3 was grown in M9 minimal media containing $^{15}NH_4Cl$ (1 g/liter, Cambridge Stable Isotopes, Andover, MA) as described in (29). The remainder of the purification of ^{15}N SUMO-E3 and E3 was the same as described above.

Two-dimensional ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) experiments were collected at 600 MHz on a Bruker Avance II

spectrometer outfitted with a cryoprobe at 298 K using WATERGATE for solvent suppression (41). A uniformly ^{15}N-labeled E3 sample was prepared by extensive buffer exchange into 20 mM Phosphate and 100 mM NaCl with a pH of 7.4 with no correction following the addition of 10% D_2O. The protein concentration in the sample used for NMR experiments was 0.1 mM and 32 scans were collected for each t1 increment with a total of 64 t1 increments.

Liquid chromatography-tandem mass spectrometry and data interpretation. Five ng of purified E3 was treated under reducing conditions (12.5 mM DTT (Sigma Aldrich, St. Louis, MO) and 125mM Ammonium Bicarbonate (Mallinckrodt Baker Inc, Paris, KY)) and non-reducing conditions (PBS) for 2 hours at 37°C. Free cysteines were alkylated in 10mM iodoadetamide for 90 min at 27°C. Samples were incubated in chymotrypsin (Sigma Aldrich, St. Louis, MO) for 18 h at 37ºC. The reactions were quenched with 1% formic acid (Sigma Aldrich, St. Louis, MO).

Six microliters of digested protein was loaded onto a C-18 reversed-phase trapping column (15 mm, 100 micron ID capillary packed with 5 micron Magic C18AQ particles with 200 angstrom pore size, Michrom Bioresources, Auburn, CA) and washed with about 20 microliters solvent A (3% acetonitrile, 0.1% formic acid). Peptides were separated by elution through a 15-cm reversed-phase nanoLC column (75 micro ID capillary pulled to a tip and packed with 5 micron Magic C18AQ particles with 100 angstrom pore size, Michrom Bioresources) by increasing solvent B (0.1% formic acid in acetonitrile) from 5% to 40% at 250

ml/min over 30 minutes and electrosprayed directly into the source of an ion trap mass spectrometer which recorded mass spectra and data-dependent tandem mass spectra of the peptide ions (LCQ Deca XP, ThermoFinnigan, San Jose, CA). Data dependent tandem mass spectra were recorded by acquiring a precursor mass spectrum followed by two tandem mass spectra of the two most intense ions from the precursor scan (unless excluded by the dynamic exclusion algorithm in which case the next most abundant ions were selected). Spectra were either automatically interpreted using the database searching tool Mascot v. 1.9 (Matrix Science, Boston, MA) and manually validated or manually interpreted (as for peptides containing disulfide bonds).

Generation of mutant virus stocks. Cysteine and PPCXPCC motif mutations in TE12 cDNA clones were introduced using Quikchange Site-Directed Mutagenesis (Stratagene, La Jolla, CA) and the genes corresponding to the E3 and E2 proteins were sequenced. Mutant cDNA clones were linearized with *Sac*I and in vitro transcribed with SP6 RNA polymerase (40). For electroporation, ~10^8 BHK cells were trypsinized, washed twice in PBS and resuspended in a final volume of 500 µl PBS. Cells were combined with in vitro transcribed RNA in a 2mm gap cuvette and were pulsed once at 1.5 kV, 25 µF, 200 Ω using BioRad Gene Pulser Xcell Electroporation System. After a 10 minute recovery at room temp, cells were diluted 1:10 in MEM/10% FBS. Virus was harvested ~24 hpe and titer was determined using standard plaque assay procedure (40).

Immunofluorescence staining for E2 expression. BHK-21 cells were transfected with viral RNA using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturers protocol. To view E2 expression at the plasma membrane, cells were fixed with 1.5% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) 6 hours post-transfection for 20 minutes at room temperature. Following fixation, cells were washed with 1×PBS and stained with a polyclonal antibody against E2 (Cocalico, Reamstown, PA) and the secondary antibody Alexa Flour 488 (Invitrogen, Carlsbad, CA). Nuclei were stained with 10μg/ml DAPI (Sigma Aldrich, St Louis, MO). To view E2 expression in the cytoplasm, BHK cells were fixed and permeablized in 1.5% paraformaldehyde and 0.01% Triton X-100 at 10 hours post-transfection for 20 minutes at room temperature and stained for E2 and DAPI. All washes and incubations were performed with 1×PBS+0.01% Triton X-100 at room temperature.

Revertant Screening. Mutant viruses were serially passaged over BHK cells and plaque size was monitored to indicate a potential second-site revertant. Genomic RNA was isolated from large plaques that had been passaged once (40). RT-PCR was performed on the region of the genome corresponding to the structural proteins and mutations were identified. In order to verify that the larger plaque phenotype was due to the specific mutation, the revertant site was introduced into the mutant virus and plaque size and growth kinetics between the mutant virus, the isolated revertant, the mutant virus containing the revertant site, and wild-type virus were determined.

RESULTS

Expression, Purification and Characterization of Recombinant E3 protein.

To characterize the properties of E3, we recombinantly expressed the protein in bacteria. The glycosylation site on Sindbis E3 was shown to be dispensible for virus production (Melki and Mukhopadhyay, data not shown), consistent with other deglycosylation mutants (42). E3 was expressed and purified as a His-tagged SUMO fusion. Incubation of the SUMO-E3 protein with a SUMO specific protease cleaved the E3 protein from the SUMO-E3 protein (Figure 2A, inset) at the first residue of the E3 protein. Gel filtration of the recombinant E3 protein on a Superdex peptide column showed the protein elutes primarily as a single peak at an elution volume corresponding to a ~7kDa protein (Figure 2A) that was confirmed as E3 by mass spectrometry analysis.

E3 is predicted to have a high alpha-helical content (Figure 2B). This was confirmed experimentally using far-UV circular dichroism (CD) (Figure 2C). To evaluate the tertiary structure of Sindbis E3, an HSQC spectrum (^{15}N heteronuclear single quantum coherence) was obtained. The spectrum showed monodisperse peaks with good chemical shift dispersion suggesting a monomeric, folded protein (Figure 2D). Approximately 10% of the chemical shifts are consistent with random-coil values, indicating part of the E3 protein is unstructured. NMR lineshape analysis indicated similar linewidths for the majority of crosspeaks indicating that the E3 protein was predominantly in a single oligomeric state (Figure 2D), consistent with gel filtration (Figure 2A).

Identification of Disulfide Bonds in Recombinant E3. There are four conserved Cys residues in alphaviruses (Figure 1). In Sindbis virus, these residues are Cys10, Cys19, Cys25 and Cys56. To assess whether these cysteines were involved in disulfide bond formation, we used chemical modification followed by LC-MS/MS. We treated E3 under native (no DTT) and reduced (12.5 mM DTT) conditions, coupling free thiols with iodoacetamide, digesting the protein with chymotrypsin and then examining the peptides by LC-MS/MS. Analysis of the data (an example is shown in Figure 3), confirms the presence of a disulfide bond between Cys19 and Cys25 in the Sindbis E3 protein. Under native conditions we observed the peptide GNSFPCDRPPTCY (covering residues 14-26) was not alkylated indicating Cys19 and Cys25 were buried or formed an intrapeptide disulfide. Based on the mass of the peptide, the latter is favored. Peptides containing Cys10 and Cys56 show these residues were alkylated, suggesting they do not form disulfide bonds in E3. In contrast, when E3 was incubated under reducing conditions, Cys19 and Cys25 were alkylated. Data of similar quality (not shown) has also been obtained for the peptide covering residues 12-26 and also verifies the presence of a disulfide bond between Cys19 and Cys25.

A small percentage of the sample shows Cys10 and Cys56 form a disulfide bond but this is not the predominant species and, as shown below, is not biologically relevant.

Mutational Analysis of Cysteine Residues in Sindbis Virus. Possible roles for the Cys19 and Cys25 residues are (1) to form a structurally essential disulfide bond, (2) have an enzymatic/functional role during virus assembly, or (3) both. To investigate the role of Cys19 and Cys25 in virus assembly, Cys mutations were made in Sindbis virus and infectivity was determined (Table 1). Cys was substituted to both Ser and Ala to ensure the phenotype observed was not due to the nature of the amino acid or its size. Both amino acid substitutions showed similar trends (Table 1). Single site mutations of Cys19 and Cys25 resulted in virus with lower titers and small plaque size compared to wild-type virus, indicating the presence of at least one of these two cysteines is important in virus assembly but both are not essential. The double mutant, Cys19 and Cys25, was not viable and no infectious virus was recovered. Immunofluorescence assays indicated E2 protein was being translated but not transported to the cell membrane in the double mutants (data not shown).

As a control, the C56S mutant, which does not form a disulfide bond in recombinant Sindbis E3 had infectivity levels comparable to wild-type virus. The Cys10 mutants had a lower titer compared to wild-type, but these mutations change the ER signal sequence reducing the total amount of spike proteins and virus particles being synthesized.

It is interesting that a single site Cys mutation still yielded infectious particles suggesting the virus utilizes alternative mechanisms for disulfide bond formation during assembly. This alternative pathway(s), however, is not sufficient when both Cys19 and Cys25 are mutated.

Characterization of recombinant E3 protein containing the PPCXPCC Motif. Our results indicate that Cys residues in E3 have a functional rather than structural role. In a sub-set of alphaviruses, the E3 protein contains a Pro-Cys rich region, the PPCXPCC motif. We examined the effect of each Cys residue in the PPCXPCC motif on disulfide bond formation by generating Sindbis E3 mutants that contained the entire PPCXPCC motif or part of the motif (Figure 4 and Table 2). Sindbis E3+PCV+C contained the entire PPCVPCC motif and introduced two additional Cys residues. Sindbis E3+PCV and Sindbis E3+C each contained part of the motif with one additional Cys residue. To keep the amino acid numbering consistent, Sindbis E3 numbering is used. To indicate the inserted "PCV" residues in SINV+PCV and SINV+PCV+C, these are referred to as insP, insC, and insV and follow residue Pro22 in Sindbis E3. An amino acid substitution of T24C was made to generate the "+C" component in SINV+C and SINV+PCV+C mutants and this is referred to as Cys24.

The recombinant proteins expressed to similar levels as Sindbis E3 and were purified in a similar manner. The disulfide bonds in these E3 proteins were determined as for recombinant Sindbis E3. The pattern of disulfide bonds in Sindbis E3 and the PPCXPCC mutants can be divided into two groups: sequential and nested disulfide bonds (Figure 4). SINV+PCV+C and SINV+C both had two sets of sequential disulfide bonds; Cys19-insC and Cys24-Cys25 in SINV+PCV+C, and Cys10-Cys19 and Cys24-Cys25 in SINV+C (Figure 4). In

contrast, SINV+PCV contained nested disulfide bonds, Cys10-Cys25 and Cys19-insC (Figure 4).

The insertion of a single Cys residue (T24C), present in SINV+PCV+C and SINV+C, did not disrupt protein function but rather induced the formation of a disulfide bond between two adjacent Cys residues (Cys24-Cys25). While vicinal disulfides are rare, these bonds are not unprecedented (8, 9, 19, 20, 48) and energetically acceptable (53). Other proteins with vicinal disulfide bonds are involved in redox processes or are regulated by redox events, suggesting this bond is transient as pE2 travels through the secretory system.

Role of the PPCXPCC motif in virus. In order to determine if the disulfide bond pattern seen in E3 correlated with infectivity of mutant virus, the SINV+PCV+C, SINV+PCV, and SINV+C mutations were made in Sindbis virus (Table 2). SINV+PCV+C and SINV+C had titers comparable to wild-type SINV indicating Cys24 by itself does not play a critical role in virus assembly. However, insertion of PCV alone resulted in very little to no infectious virus particles being released. Comparing the in vivo infectivity results with the disulfide bond pattern in the E3 protein, all the mutants that are viable in vivo have disulfide bond patterns that contain 1 or more sequential disulfide bonds. The SINV+PCV mutant is not viable and the E3 protein containing this mutation consists of two disulfide bonds that are nested one within another (Figure 4). Immunofluorescence assays which showed pE2 was expressed but very low amounts were transported to the plasma membrane (Figure 5). These results were confirmed by pulse-chase

experiments which should the amount of E2 at the cell surface of PCV transfected cells is severely reduced compared to wild-type virus (data not shown). Improper folding of pE2, a result of misfolded E3, would severely impact the heterodimerization between pE2 and E1.

Isolation and Identification of a Second-Site Revertant for SINV+PCV. The yield of SINV+PCV infectious particles was attenuated and the plaque phenotype was small. Serial passaging of this mutant virus over BHK cells led to isolation of a large plaque that was comparable in size to wild-type Sindbis virus. RNA sequence from the isolated large plaque revealed a second-site reversion at position E3 T24P (Table 2) creating PPCVPPC in contrast to PPCVPTC found in SINV+PCV. This revertant grew at titers comparable to wild-type Sindbis virus and the cellular localization of pE2/E2 was similar to wild-type Sindbis, SINV+PCV+C, and SINV+C virus (data not shown). When the T24P second-site revertant site was introduced into SINV+PCV virus (named SINV+PCV+P, Table 2), this mutant grew to a wild-type titer, confirming that this single residue could enhance viral infectivity.

The disulfide bond pattern of the SINV+PCV+P E3 protein was determined and two sequential disulfide bonds were identified. Cys10 and Cys19 formed one bond while residues insC and Cys25 formed another (Figure 4). As seen with other infectious mutants, sequential disulfide bonds were present.

The reversion of T24P now creates a PPCXPPC sequence, in contrast to PPCXPCC, in the virus suggesting the Pro-Pro residues may be a structural substitute for the vicinal disulfide bonds seen in SINV+C and SINV+PCV+C. The corresponding T→P mutation was made in Sindbis virus and surprisingly this mutant, SINV+P, grew like wild-type virus (Table 2) even though the E3 protein now contained three consecutive Pro residues.

DISCUSSION

E3 is a key player in alphavirus spike assembly but is not required for viral entry. Although E3 had previously been isolated from the media of virus infected cells (32, 49), the protein was denatured during the purification and, as a result, no structural information about E3 was obtained. In this study, by using a combination of mutant viruses and recombinant E3 protein, fundamental structural information about E3 was obtained. Analysis of these results allows us to hypothesize on a possible function of the E3 protein as discussed below.

E3 is a small, extracellular protein that contains multiple conserved Cys residues. Two Cys residues, Cys19 and Cys25, in recombinant Sindbis E3 are involved in disulfide bond formation. E3 lacking either Cys19 or Cys25 had reduced infectivity and if both residues were deleted, the virus was non-infectious. It is interesting to note that the C→S and C→A single-site mutants both had attenuated titers compared to wild-type virus, but the C→S mutations were more severe. One explanation is by inserting a polar residue into the putative functional/catalytic site of E3 prevents proper protein association

between E3 and its functional partner. However, inserting a more hydrophobic Ala, which charge-wise is more similar to Cys, does not disrupt of interfere with this interaction as much.

Mutant E3 proteins that contained the PPCXPCC motif found in some alphaviruses also had a mix of oxidized and reduced Cys residues. Furthermore, there was a correlation with the pattern of disulfide bonds and infectivity of the mutant virus. E3 proteins that contained single or sequential disulfide bonds (Sindbis, SINV+PCV+C, SINV+C, SINV+PCV+P) produced infectious virus particles at wild-type titers. E3 proteins (SINV+PCV) that contained nested disulfide bonds did not produce infectious particles. Taken together, our results suggest E3 has a functional role in virus assembly because deletion, site-directed mutagenesis, and insertion of Cys residues into a specific region of E3 do not abolish virus assembly.

We hypothesize that the E3 protein is a viral protein disulfide isomerase that catalyzes the proper folding and disulfide bond formation in pE2/E2. E3 has labile disulfide bonds and is in close proximity to E2 throughout the assembly pathway. E2 contains 14 Cys residues in the ectodomain, presumably most of which form disulfide bonds. Assembly of spikes is a series of steps that occur in different pH environments. The reshuffling of disulfide bonds in pE2 (34), a possible result of the putative disulfide isomerase activity of E3, could be a key regulatory mechanism for spike formation. If E3 was functioning as a disulfide isomerase by forming intermolecular disulfide bonds with E2 during assembly, then the absence of one cysteine residue (Cys19 or Cys25) might not be as

detrimental as the loss of both residues.

The rationale for a virus providing its own disulfide isomerase is to catalyze a reaction that occurs naturally, albeit slower, in the host. It has been shown that along with PDI, calreticulin, calnexin, and ERp57 are all involved as chaperones in spike formation (33, 34, 36-38) suggesting spike formation is a complex process which requires tremendous regulation during folding, heterodimerization, and assembly. By acting as a putative protein disulfide isomersae, E3 aids in folding the large quantity of E2 that is produced during a viral infection. It is known that if pE2 is not present in the ER, E1 will be degraded and will not be transported to the membrane. E3 ensures that pE2 is present so that the pE2-E1 heterodimerization can occur. E3 is cleaved in the trans-Golgi only after heterodimerization of pE2 and E1, trimerization of the heterodimers, and spike assembly are completed. After this, E3 is dispensable.

Aside from other alphavirus E3 proteins, there are no known proteins that are similar to E3 based on amino acid sequence. Inspection of the E3 sequence reveals a Cys-X-X-Cys in the subset of alphaviruses that contain the PPCXPCC motif. This Cys-X-X-Cys sequence is shared by many redox proteins, including protein disulfide isomerase. Despite lacking any sequence similarity with one another, all of the Cys-X-X-Cys motif containing proteins interact with cysteine-containing substrates (31, 51, 52). Further studies have shown that a Cys-Gly-Cys tripeptide is an efficient catalyst of disulfide isomerization despite not containing the Cys-X-X-Cys motif (50). Alphaviruses that do not contain the PPCXPCC motif do have a conserved Cys-X-X-Pro-Pro-X-Cys sequence, and

putative protein disulfide isomerase activity may involve this sequence. Many non-viral proteins have Cys residues separated by 0-5 amino acids. These proteins include disulfide isomerases and redox sensitive proteins (53). Like the unexpected Cys-Gly-Cys disulfide isomerase activity results, the putative disulfide isomerase activity of the Cys-X-X-Pro-Pro-X-Cys sequence is not known until experimentally tested. These experiments are underway.

E3 is conserved in the alphavirus genome. If its sole purpose was to transport the E3-E2-6K-E1 polyprotein into the ER during folding, the signal sequence from E3 could have been integrated into E2 long ago. It is also clear that cleavage of E3 from E2 is required to form fusion competent spikes. The hypothesis that E3 may function as a disulfide isomerase in the folding of E2 during assembly is consistent with the previous biochemical genetic results as well as the data presented here.

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FIGURE LEGENDS

FIGURE 1. E3 amino acid sequence alignment from a representative group of alphaviruses. The cysteines marked with asterisks are conserved in all alphavirus species. The ◊ indicates the conserved but non-essential glycosylation site. The PPCXPCC motif present in ~50% of alphaviruses is underlined. SINV=Sindbis, SFV=Semliki Forest, RRV=Ross River, BFV=Barmah Forest, EEE=Eastern Equine Encephalitis, ONN=O'nyong nyong, IGB=Igbo Ora, OCK=Ockelbo, WEE=Western Equine Encephalitis, AUR=Aura, VEE=Venezuelan Equine Encephalitis.

FIGURE 2. Expression and Characterization of Sindbis E3 protein. (A) Gel filtration profile of Sindbis E3. E3 was eluted from a Superdex peptide HR 10/30 column at a flow rate of 0.5 ml/min. Comparison with a calibration curve shows a majority of the protein elutes at ~7kDa, the size of an E3 monomer. *Inset:* SDS-PAGE showing concentrated Sindbis E3 after Superdex peptide column. Molecular weight of E3=7kDa. (B) Amino acid sequence of Sindbis E3 protein. The N-terminal signal sequence that is responsible for trans-localization of E3-E2-6K-E1 to the lumen of the ER is underlined, the conserved glycosylation site is indicated by the arrow, and the four cysteine residues conserved in all alphaviruses are marked with asterisks. Cylinders represent regions of predicted alpha helices as determined by JPred (10). (C) Representative CD spectrum of purified Sindbis E3 protein. Purified E3 was prepared in 20 mM phosphate buffer pH 8 and 100 mM NaF and an average of 3 spectra from 190 nm to 260 nm were

recorded in a 1 mm path length cuvette. The minima at 208 and 220 nm indicate alpha helical secondary structures, consistent with secondary structure prediction (Figure 2C). (D) ^{1}H-^{15}N-HSQC of Sindbis E3. Proton-nitrogen NMR correlation spectrum of 100 μM E3 at 600 MHz, 25°C, pH 7.5 (20 mM PO_4 buffer, 300 mM NaCl). The well-dispersed chemical shifts indicate that E3 is well-folded. The linewidths are typical for a monomeric globular protein of the size of E3, which is consistent with gel filtration results (Figure 2A).

FIGURE 3. An example of disulfide bond identification as determined by chemical modification and LC-MS/MS analysis. Mass spectrometry analysis of the peptide GNSFPCDRPPTCY (covering residues 14-26) of Sindbis E3 under non-reduced conditions is shown in panels (A, C) and under reduced conditions is shown in panels (B, D). Under non-reduced conditions, the m/z for the doubly-charge GNSFPCDRPPTCY precursor peptide is 777.7. The same peptide under reduced conditions has a doubly-charge m/z of 835.6. Initial base peak chromatograms showed good separation for both the non-reduced and reduced samples. Furthermore, selected ion chromatograms showed a distinct peak corresponding to each precursor peptide (data not shown). Data dependent tandem mass spectra were recorded by acquiring a precursor mass spectrum followed by two tandem mass spectra of the two most intense ions from the precursor scan. (A) Mass spectrum showing the intact precursor under non-reduced conditions with m/z 777.7 and (B) under reduced conditions with m/z 835.6. In both spectra the precursor is the main component indicating no

other peptide fragments were co-eluted. (C) The tandem mass spectrum of the m/z 777.7 precursor (non-reduced samples) and (D) the m/z 835.6 precursor (reduced samples). The sequence-specific fragment ions in both (C) and (D) are labeled y5-y11 and all match the calculated m/z values consistent with the disulfide bond being retained in these fragments (C) or with the absence of a disulfide bond and the two Cys residues being alkylated (D).

FIGURE 4. Disulfide bonds present in E3 PPCXPCC motif mutants determined by chemical modification and LC-MS/MS analysis. Disulfide bonds between Cys residues in the E3 protein of the PPCXPCC motif mutants are indicated with a "‿". A horizontal line is drawn over the PPCXPCC motif, the four conserved cysteines in E3 are in red, and portions of the PPCXPCC motif that were introduced into Sindbis E3 are shown in green. The second-site reversion site, T24P, isolated from the SINV+PCV mutant is shown in blue in the sequence marked SINV+PCV+P. Each E3 protein was processed and analyzed as described in Figure 3 and in the text.

FIGURE 5. Expression of pE2 and E2 protein in SINV+PCV. BHK-21 cells were transfected and after 8 hours fixed for intracellular staining (top) with 1.5% paraformaldehyde + 0.01% Triton X 100 or cell surface staining (bottom) with 1.5% paraformaldehyde. Cells were stained with an antibody recognizing E2. Cells intracellularly stained were imaged at 100x to show the internal staining

pattern. Cells surface stained were imaged at 40x to show a larger distribution of cells.

1 **TABLE 1**

2

TABLE 1. Infectivity of Sindbis E3 Cysteine Mutations[a]

Virus	Titer (PFU/ml)	Plaque Size (mm) after 24 hours
WT SINV	4.5×10^9	4
C56S	6.2×10^9	4
C19S	1.9×10^5	2
C19A	2.8×10^8	1
C25S	7.2×10^4	2
C25A	4.5×10^7	1
C19S & C25S	No infectious particles	--
C19A & C25A	No infectious particles	--

[a]BHK-21 cells were electroporated with equal amounts of in vitro transcribed RNA. Virus was harvested 24 hpt and titered by plaque assay.

1 **TABLE 2**

2

TABLE 2. Infectivity of PPCXPCC Motif Mutants[a]

Virus	Mutation	Titer (PFU/ml)
WT SINV	14 NVSFPCDR**P---PTC**YTREPSRAL 34	8×10^9
PPCXPCC Mutants:		
SINV+PCV+C	14 NVSFPCDR**PPCVPCC**YTREPSRAL 37	1×10^{10}
SINV+C	14 NVSFPCDR**P---PCC**YTREPSRAL 34	3×10^9
SINV+PCV	14 NVSFPCDR**PPCVPTC**YTREPSRAL 37	$<1 \times 10^1$
SINV+PCV Revertant:		
SINV+PCV+P	14 NVSFPCDR**PPCVPPC**YTREPSRAL 34	3×10^8
T24P in WT SINV:		
SINV+P	14 NVSFPCDR**P---PPC**YTREPSRAL 37	7×10^9

[a]BHK-21 cells were electroporated with equal amounts of in vitro transcribed RNA. Virus was harvested 24 hpt and titered by plaque assay.

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Figure 1

```
                 *   ◊    *        *                                *
SINV  SAAPLVT-AMCLLGNVSFPCDRP---PTCYTREPSRALDILEENVNHEAYDTLLNAILRCGSSGRSKR 64
SFV   -SAPLIT-AMCVLANATFPCFQPPCVPCCYENNAEATLRMLEDNVDRPGYYDLLQAALTCRNGTRHRR 66
RRV   -SAALM---MCILANTSFPCSSPPCYPCCYEKQPEQTLRMLEDNVNRPGYYELLEASMTCRNRSRHRR 64
BFV   SAAALXITALCVLQNLSFPCDAPPCAPCCYEKDPAGTLRLLSDHYYHPKYYELLDSTMHCPQGRRPKR 68
EEE   ---SLAT-VMCVLANITFPCDQPPCMPCCYEKNPHETLTMLEQNYDSRAYDQLLDAAVKCNAR-RTRR 63
ONN   -SLALP--VMCLLANTTFPCSQPPCAPCCYEKKPEETLRMLEDNVMQPGYYQLLDSALACSQR-RQKR 64
IGB   -SLALP--VMCLLANTTFPCSQPPCAPCCYEKKPEETLRMLEDNVMQPGYYQLLDSALACSQH-RQRR 64
OCK   SAAPLVT-AMCLLGNVSFPCNRP---PTCYTREPSRALDILEENVNHEAYDTLLNAILRCGSSGRSKR 64
WEE   ----LVT-ALCVLSNVTFPCDKP---PVCYSLAPERTLDVLEENVDNPNYDTLLENVLKCPSR-RPKR 59
AURA  --SRAIT-AMCILQNVTFPCDRP---PTCYNRNPDLTLTMLETNVNHPSYDVLLDAALRCPTR-RHVR 61
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```
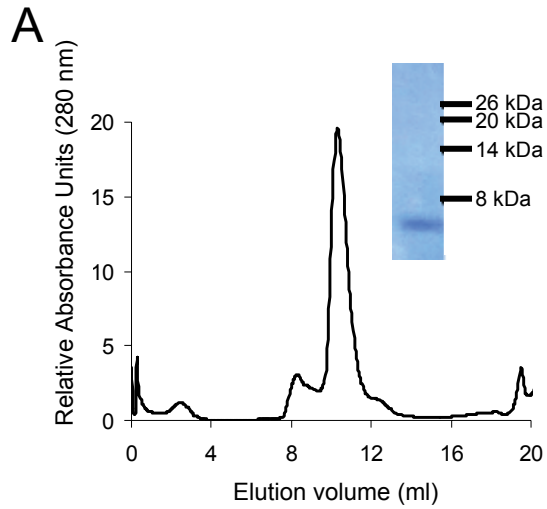

Figure 2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Elution volume (ml)
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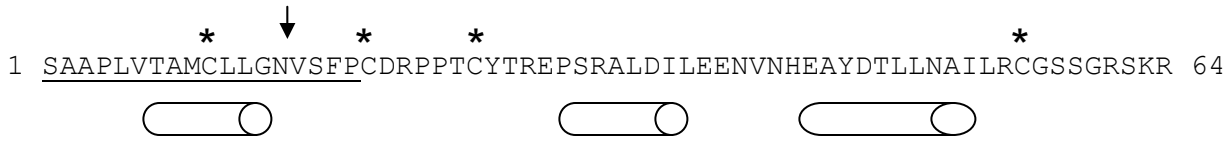
20 kDa
14 kDa
8 kDa

B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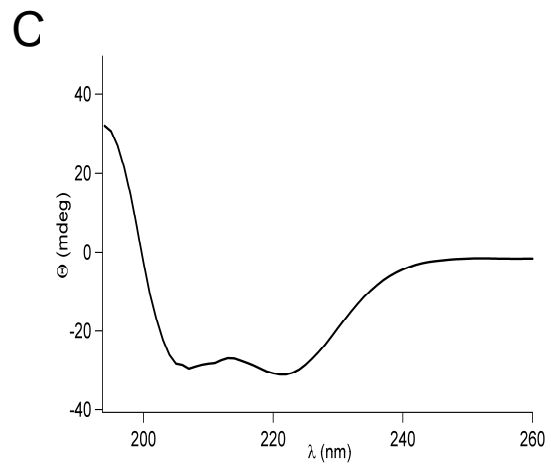
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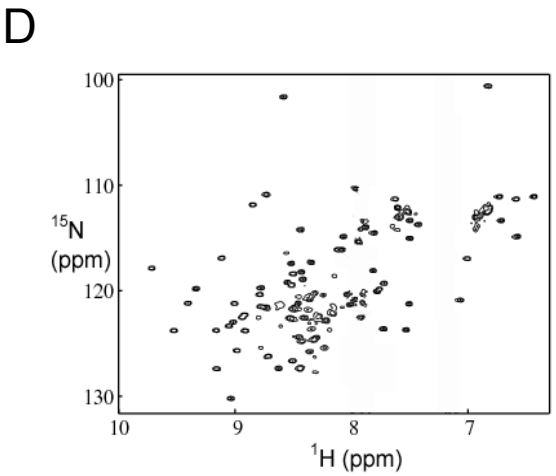
260
λ (nm)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(ppm)
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7
1H (ppm)

Figure 3

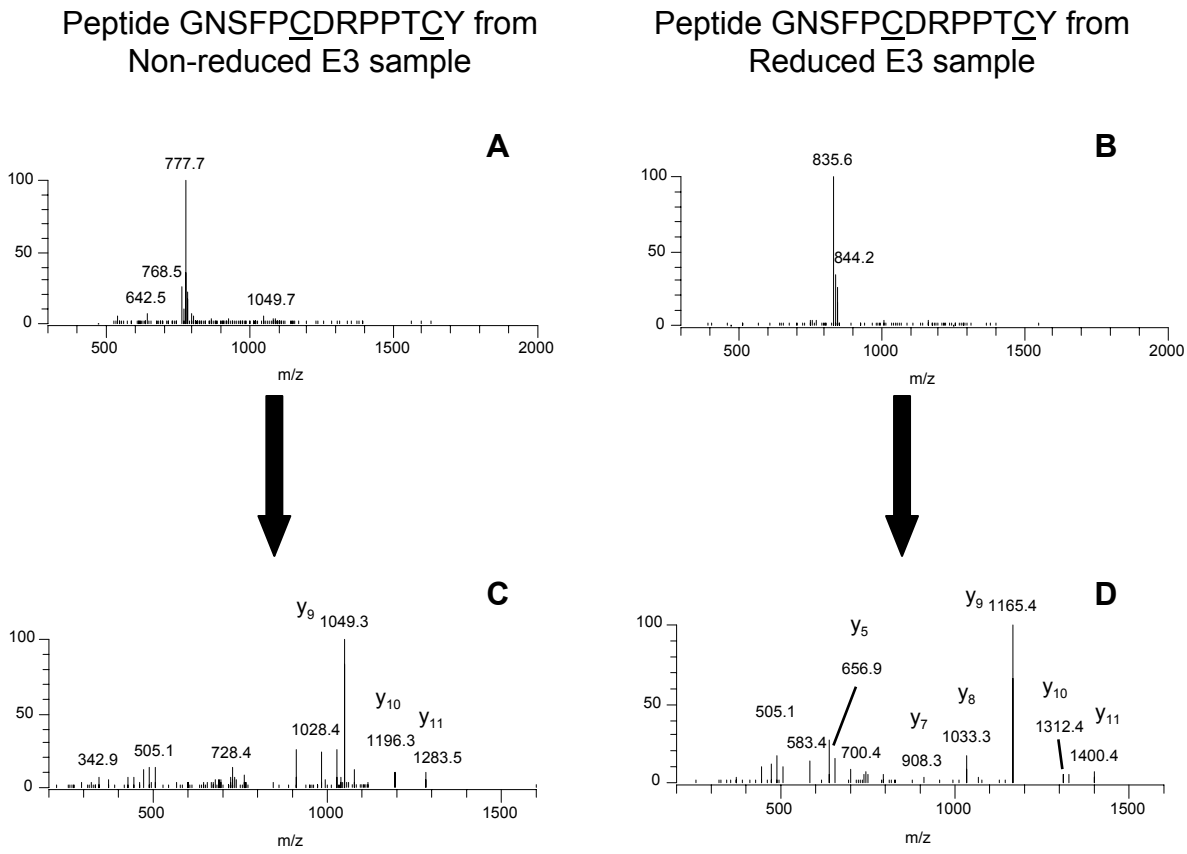
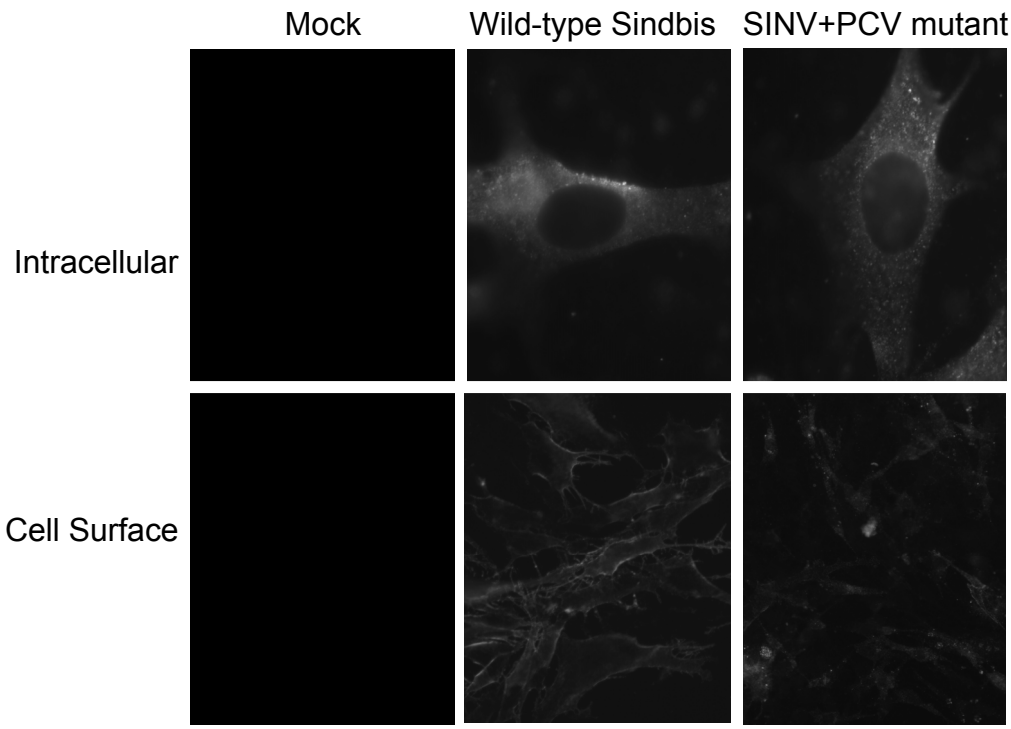

Peptide GNSFPCDRPPTCY from Non-reduced E3 sample
Peptide GNSFPCDRPPTCY from Reduced E3 sample
A
777.7
768.5
642.5
1049.7
m/z
B
835.6
844.2
m/z
C
y9 1049.3
y10
y11
1028.4
1196.3
1283.5
342.9
505.1
728.4
m/z
D
y9 1165.4
y5
656.9
y8
y10
505.1
y7
y11
583.4
1033.3
1312.4
700.4
908.3
1400.4
m/z

Figure 4


SINV
SAAPLVTAMCLLGNVSFPCDRP---PTCYTREPSRALDILEENVNHEAYDTLLNAILRCGSSGRSKR
SINV+PCV+C
SAAPLVTAMCLLGNVSFPCDRPPCVPCCYTREPSRALDILEENVNHEAYDTLLNAILRCGSSGRSKR
SINV+C
SAAPLVTAMCLLGNVSFPCDRP---PCCYTREPSRALDILEENVNHEAYDTLLNAILRCGSSGRSKR
SINV+PCV
SAAPLVTAMCLLGNVSFPCDRPPCVPTCYTREPSRALDILEENVNHEAYDTLLNAILRCGSSGRSKR
SINV+PCV+P
SAAPLVTAMCLLGNVSFPCDRPPCVPPCYTREPSRALDILEENVNHEAYDTLLNAILRCGSSGRSKR

Figure 5


Mock
Wild-type Sindbis
SINV+PCV mutant
Intracellular
Cell Surface